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SHORT COMMUNICATION

Both Pre-S1 and S Domains of Hepatitis B Virus Envelope Proteins Interact with the Core Particle

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The three envelope proteins of the hepatitis B virus (HBV) are encoded by a single open reading frame in the genome containing three separate in-phase AUG codons. This organization defines three protein domains (pre-S1, pre-S2, S) which form the small (S), middle (M, pre-S2/S), and large (L, pre-S1/pre-S2/S) proteins. Mature virions are generated by the budding of preformed nucleocapsids through endoplasmic reticulum (ER) membranes containing S and L proteins, whereas the M protein is not necessary. This suggests an important function for the pre-S1 domain. To investigate the protein–protein interactions involved during the maturation process of the HBV virion, we studied *in vitro* the binding affinity to purified HBV core particles of various synthetic peptides identical to regions of the envelope proteins. Data previously obtained with deletion mutants were confirmed and refined. The 13 C-terminal amino acids of pre-S1 bound efficiently to core particles, whereas other pre-S domains did not. Moreover, the amino acid sequence 56–80 in the cytosolic loop of S bound efficiently to the HBV core. This double interaction between the HBV capside and both S and pre-S1 domains may be required for virion morphogenesis.

Hepatitis B virus (HBV) is an enveloped DNA virus of the *Hepadnavirus* family which causes a variety of acute and chronic liver diseases in humans (reviewed in Ref. *1* and *2*). Its genome is encapsidated with a virus-encoded polymerase in a 27-nm diameter nucleocapsid surrounded by a host-derived lipid envelope bearing three viral surface proteins. The mature 42-nm virion is formed by a preformed cytosolic nucleocapsid interacting with surface proteins anchored in a pre-Golgi membrane and is secreted by the constitutive secretory pathway (*3–5*). The three HBV surface proteins are translated from a single open reading frame of the viral genome from three different in-phase start codons. Therefore the 226 amino acid sequence of the small (S) protein is repeated at the C termini of the middle (M) and the large (L) surface proteins, which carry the additional N-terminal 55 amino acid pre-S2 domain and the 163 amino acid (subtypes ayw) pre-S2 plus pre-S1 domain, respectively (*6*). In addition to the virion, HBV surface proteins can also be independently secreted from cells as 22-nm diameter spherical or tubular subviral particles. These empty particles are found at much higher serum concentrations than virions and consist of host lipids and viral envelope proteins without any other viral component. S protein is the major constituent of both virion and empty particle envelopes (*6*). L protein is necessary for virion maturation, and S is required but not sufficient (*3, 7, 8*). M seems to be dispensable (*3, 7*). S protein is unique among viral envelope proteins in its capacity to self-assemble with host-derived lipids into secreted empty envelope particles (*9*). It has a type I signal at its N terminus and an internal type II signal which result in the protein to traverse the membrane at least twice and form a cytosolic loop and a luminal domain (reviewed in Ref. *10*). The C-terminus of S is very hydrophobic and is believed to be embedded in the lipid bilayer (Fig. 1). L protein can adopt two different trans-membrane topologies (*11–13*). The first called e-pre-S has pre-S domains on the luminal side of the ER. After budding, pre-S domains become exposed on the surface of secreted virions and capable of binding to a virus receptor (*14*). The second called i-pre-S has the pre-S domain on the cytosolic side of the ER (Fig. 1) thus providing a specific interaction with HBV nuceocapsid during virion assembly (*7*). This specific interaction has been investigated by testing the ability of various truncated L mutants to form virions. Thus, the 17 C-terminal amino acids of the pre-S1 domain (aa 91 to 108 in the ayw subtype) are essential for virion formation, whereas the N-terminal 5/6 of the pre-S1 sequence are not required for this process (*7*). The S protein is also necessary for virion maturation but its exact function has not been determined. It is unclear whether S interacts

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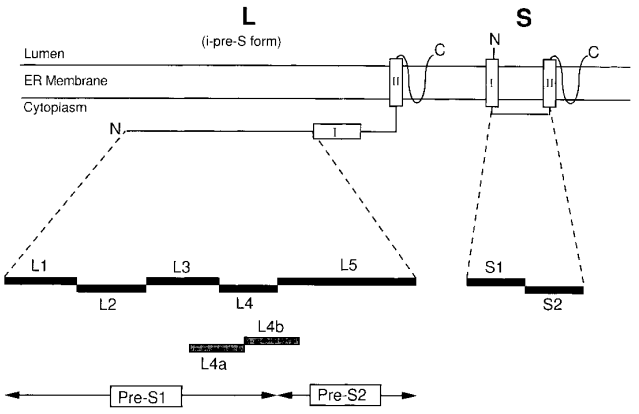

L1	1-28	MGQNLSTSNPLGFFPDHQLDPAFRANTA
L2	29-56	NPDWDFNPNKDTWPDANKVGAGAFGLGF
L3	57-85	TPPHGGLLGWSPQAQGILQTLPANPPPAS
L4	86-108	TNRQSGRQPTPLSPPLRNTHPQA
L4a	76-95	TLPANPPPASTNRQSGRQPT
L4b	96-116	PLSPPLRNTHPQAMQWNSTTF
L5	169-163	MQWNSTTFHQTLQDPRVRGLYFPAGGSSSGTVNPVLTTASPLSSIFSRIGDPALN
S1	31-55	SLDSWWTSLNFLGGTTVCLGQNSQS
S2	56-80	PTSNHSPTSCPPTCPGYRWMCLRRF

FIG. 1. Amino acid sequence (ayw subtype) of the 9 synthetic peptides covering HBV S and L surface protein domains exposed on the cytosolic side of the ER. The small HBV surface protein (S, 226 aa) spans the membrane at its N-terminal type I and the central type II signals (open boxes) and probably twice at its hydrophobic C-terminal region. S exposes a loop of 50 amino acids at the cytosolic side of ER, covered by peptides S1 and S2. The large HBV surface protein (L) contains the 226 aa of S plus 163 aa called the pre-S domain at its N-terminus. This pre-S domain has two subdomains: pre-S1, containing 108 aa (peptides L1, L2, L3, and L4); and pre-S2, containing 55 aa (peptide L5). The L protein adopts two membrane topologies (*11*): the e-pre-S L form (not shown in this figure) displaying the N-terminal pre-S domain at the ER lumen, and the i-pre-S L form (presented in the figure), exposing the pre-S domain and the N-terminal part of the S domain of L up to signal II on the cytosolic side of ER.

with L, with the HBV core or with both during particle assembly. In this work, pre-S1, pre-S2, and the cytosolic loop of S were mapped in synthetic peptides which were tested for their ability to bind purified liver-derived HBV core particles.

S and L peptides synthesis: Peptides corresponding to pre-S1, pre-S2, and the cytosolic loop of S were synthesized as shown in Fig. 1. The amino acid sequence was obtained from the Swiss-prot database (AC P03138), corresponding to the ayw subtype DNA sequence published by Galibert *et al.* (*15*). The cytosolic loop of S was covered by two 25 amino acid peptides, S1 and S2. The pre-S1 domain was covered by four peptides of 28, 28, 29, and 23 amino acids named L1, L2, L3, and L4, respectively. Pre-S2 was covered by a single 55 amino acids peptide, L5. Two additional peptides overlapping peptide L4 were synthesized: peptide 4a (the last 10 aa of peptide L3 plus the first 10 aa of peptide L4) and peptide 4b (the last 13 aa of peptide L4 plus the first 8 peptides of pre-S2) (Fig. 1). All peptides were synthesized with an automated peptide synthesizer (Applied Biosystems 431A) by the solid-phase procedure developed by Merrifield (*16*), with 9-fluorenyl-methoxycarbonyl-protected amino acids and *p*-hydroxymethyl-phenoxyacetic resin. After synthesis, the resin support and the side chain-protecting groups were removed by treatment with trifluoroacetic acid in the presence of scavengers. Peptides were purified by reverse-phase chromatography on C8 columns (Aquapore octyl; 20 μm; 100 by 10 mm; Applied Biosystems) and characterized by amino acid analysis (*17*).

Purification of HBV core particles: Human HBcAg-positive tissue obtained from a liver necropsy was homogenized in a gentle lysis buffer (10 m*M* Tris–HCl, pH 7.2, 150 m*M* NaCl, 0.1 m*M* EDTA, 0.1% NP-40, 0.05% 2 β-mercaptoethanol). Cell fragments were removed by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was spun on a isopycnic 24 to 64% sucrose gradient (wt/vol in 150 m*M* NaCl, 20 m*M* Tris–HCl, pH 7.4) at 22,000 rpm for 36 hr in a SW 28 swinging rotor (Beckman, Somerset, NJ). Fractions were tested for the presence of HBV core particles by HBcAg-specific enzyme-linked immunoassay (ELISA) as described elsewhere (*18*). Briefly, the IgG fraction from a selected human serum [anti-HBc positive up to the 10^{-7} dilution, anti-HBe and anti-HBs negative (all tests from Abbott lab., North Chicago, IL)] was used as the solid phase. Dynatech ELISA plates were coated with this IgG preparation (1 mg/ml) diluted 1/500 in 0.1 *M* carbonate buffer, pH 9.2, incubated overnight at 4° and then washed four times with a washing buffer [phosphate-buffered saline (PBS), pH 7.2, containing 0.1% Tween 20]. To avoid nonspecific adsorbtion, plates were coated with a 5% solution of dry nonfat milk in PBS, incubated for 1 hr at 37°, and then washed with the washing buffer. Fractions were diluted 1/500 in a dilution buffer [PBS containing 0.05% Tween and 2% milk powder] then added to the wells and incubated for 3 hr at 37°. After four washes, the plates were incubated for 1 hr at 37° with the monoclonal anti-HBc antibody 6C6A1 (*18*) diluted 1/1000 in the dilution buffer, then washed four times and incubated for 1 hr at 37° with peroxidase-conjugated goat anti-mouse IgG (Cappel, West Chester, PA) diluted 1/2000 in dilution buffer. After four washes, the peroxidase reaction was performed by adding a mixture of hydrogen peroxide/*o*-phenylenediamine substrate (Sigma, St-Louis, MO) and incubating for 30 min in the dark. The reaction was stopped with 4 *N* H_2SO_4 and the optical density at 492 nm was determined with a microplate reader.

HBc Ag-positive fractions were pooled, dialyzed against 25 m*M* Tris–HCl, pH 8.5, 50 m*M* NaCl, and the preparation was further purified by a ion-exchange chromatography on a DEAE-trysacryl *M* gel (LKB, Bromma, Sweden). A discontinuous NaCl gradient (0.15 *M*/0.3 *M*/

1 *M*) was used for elution. The total protein concentration in fractions was determined by measuring the optical density at 280 nm and HBc Ag activity assayed in 1/400 dilutions by ELISA as described above (Fig. 2). Fractions 30 to 37 were pooled and concentrated 10-fold by centrifugation at 25,000 rpm for 6 hr (4°, rotor SW 28, Beckman). This final core particle preparation contained 0.4 mg/ml of protein and the specific activity measured with 1/2 serial dilutions by HBcAg ELISA was 2^8 per mg of protein. HBV core particles of 28 to 32 nm diameter were observed by electron microscopy (Jeol 1200 EX, Tokyo) after negative staining with 2% uranyl acetate.

Binding of envelope peptides to core particles: Binding was first evaluated as the ability of the peptides to inhibit core particle capture by the HBcAg-specific immunoassay. To obtain a reproducible optical density value of 2.5 in control wells (without peptide), the assay was slightly modified as follows. Human anti-HBc IgG diluted 1/500 dilution in 50 m*M* bicarbonate buffer, pH 9.6, were coated onto polystyrene plates (Maxisorp, Nunc, Naperville, IL), for 20 hr at 4° (100 μl per well). The HBV core preparation was diluted 1/100 in 1 m*M* PBS, 15 m*M* NaCl, pH 7.4, and incubated for 20 hr at 4° with each of a series of concentrations (0 to 100 μg/ml) of each peptide in the

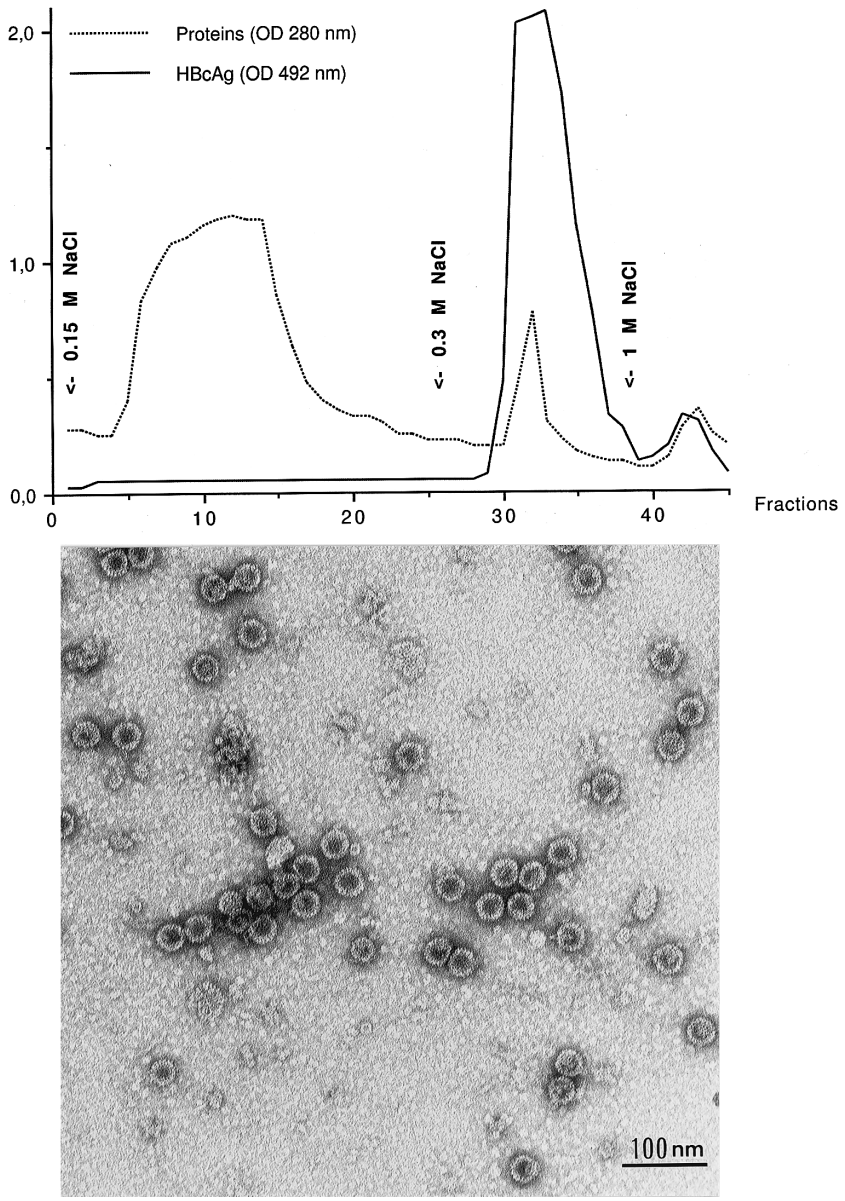

FIG. 2. Purification of HBV core particles from a human liver. Top, elution profile of the ion-exchange chromatography on DEAE-trysacryl M gel. A discontinuous NaCl gradient (0.15 *M*/0.3 *M*/1 *M*) was used for elution. Most contaminant proteins as assessed by following the optical density (OD) at 280 nm were collected at the 0.15 *M* elution. HBc Ag activity determined by a specific immunoassay (OD at 492 nm) was detected in fractions collected during the 0.3 *M* NaCl elution. Bottom, pooled and concentrated HBc Ag-positive fractions examined by negative staining under an electron microscope showing intact HBV core particles.

same buffer. Plates were washed three times with 0.01 *M* PBS, 0.15 *M* NaCl, pH 7.4, containing 0.5% Tween 20 and blocked by the addition of 300 μl of PBS containing 2% newborn calf serum (NBCS) incubated for 45 min at 37°. The mixed core particles/peptide preparations were then added to the wells and incubated for 1 hr at room temperature. After three washes, 100 μl of monoclonal anti-HBc 6C6A1 diluted 1/10,000 in 0.05 *M* PBS, 0.75 *M* NaCl, pH 7.4, containing 5% NBCS, 5% bovine serum albumin (BSA), and 0.5% Tween 20, was added and incubated for 1 hr at room temperature. Following three washes, 100 μl of horseradish-peroxidase-conjugated goat anti-mouse $F(ab')^2$ (Tago, Burlingame, CA) diluted 1/10,000 in the same buffer was added. Plates were incubated for 30 min at 37°, washed three times, and a mixture of hydrogen peroxide/*o*-phenylenediamine was added and left for 30 min in the dark at room temperature. Color development was stopped with 2 *N* H_2SO_4 and the optical density was read at 492 nm. Preliminary results obtained with the "L" peptides prompted us to synthesize two additional peptides, L4a and L4b, overlapping peptide L4. Reduction in the signal was quantified as a percentage of inhibition: 100X(1-[OD with peptide/OD without peptide]) and correlated with increasing peptide concentration. The mean percentages of inhibition obtained in four independent experiments with the nine peptides at three different concentrations (5, 50, and 100 μg/ml) were determined (Table 1).

We further tested the peptides ability to bind directly to HBV core particles by an alternative method based on peptide immobilization to the solid matrix. The direct immobilization of free peptides to the ELISA plate resulted in a complete loss of binding activity for all peptides. However, when peptides were conjugated to bovine serum albumin (BSA) with carbodiimide as previously described (*19*), a reproducible HBV core binding activity was obtained with peptides L4b and S2: Dynatech ELISA plates were coated with the different peptides coupled to BSA (or with BSA alone) at a concentration of 20 μg/ml in 0.05 *M* bicarbonate buffer (pH 9.6) for 24 hr at 4° (200 μl per well). Plates were washed three times with 0.01 *M* PBS, 0.15 *M* NaCl, pH 7.4, containing 0.5% Tween 20, and blocked by the addition of 300 μl of PBS containing 2% newborn calf serum (NBCS) incubated for 45 min at 37°. After one wash, the HBV core preparation diluted 1/50 in 1 m*M* PBS, 15 m*M* NaCl, pH 7.4, was incubated for 24 hr at 4°. The other steps for core particles detection (monoclonal anti-HBc and peroxidase-conjugated goat anti-mouse incubations) were performed as above. The cut-off value was choosen as twofold the mean value obtained from nine control wells containing BSA alone (2×0.035 in a representative experiment). Binding activity for a given peptide was expressed as the O.D. value/cut-off value (Table 2). The omission of either the core particles or the monoclonal anti-HBc resulted in O.D. values below the cut-off value for all BSA-coupled peptides.

Pre-S domains: Peptides L1, L2, and L3 covering the 85 N-terminal amino acids (80%) of pre-S1, as well as peptide L5 (all 55 amino acids of pre-S2), did not bind to HBV core particles in both assays. Peptide L4, corresponding to the 23 C-terminal amino acids of pre-S1, was found to bind to HBV core particles in the inhibition immunoassay (Table 1). Peptide L4b corresponding to the 13 C-terminal amino acids of pre-S1 plus the 8 N-terminal amino acids of pre-S2 bound efficiently to HBV core particles in the inhibition immunoassay, whereas peptide L4a had only weak binding affinity. In addition, peptide L4b was the only "L" peptide that bound to HBV core particles in the direct binding assay (Table 2). These results suggest that the 13 C-terminal amino acids of pre-S1 are involved in HBV envelope–core interaction.

Recently, Dyson and Murray (*20*) tested random hexapeptides for their binding properties to recombinant HBV core particles. Selected hexapeptides were further investigated and a peptide LDPAFR equivalent to positions 19 to 24 of pre-S1 inhibited the binding of HBV cores to the L polypeptide. In contrast, our L1 peptide (aa 1–28 of pre-S1) containing this 6 amino acids sequence did not bind to HBV core particles in our assays. This discrepancy may be due to the different lengths of the peptides

TABLE 1

Binding of HBV Envelope Peptides to HBV Core Particles Tested by Inhibition in a HBc Ag-Specific Immunoassay

	Peptides								
Concentration	L1	L2	L3	L4	L4a	L4b	L5	S1	S2
5 μg/ml	–	–	–	15 ± 3%	4 ± 2%	16 ± 5%	–	–	38 ± 4%
50 μg/ml	–	–	–	28 ± 5%	4 ± 2%	77 ± 2%	–	–	80 ± 3%
100 μg/ml	–	–	–	33 ± 2%	12 ± 4%	91 ± 2%	–	–	83 ± 7%

Note. The binding was evaluated as the peptides ability to inhibit core particle capture and detection in a specific immunoassay. OD values given by core particles alone (standardized to 2.5) were compared with OD values for core particles preincubated with various concentrations of a peptide. Results were expressed as percentages of inhibition: 100 × (1-[OD with peptide/OD without peptide]). Values reported here represent the mean percentages and standard deviations of 4 independent experiments performed with the 9 peptides at 3 concentrations (5, 50, and 100 μg/ml). –, no inhibition.

TABLE 2

Direct Binding of BSA-Coupled HBV Envelope Peptides to HBV Core Particles

Peptides								
L1	L2	L3	L4	L4a	L4b	L5	S1	S2
0.4 ± 0.05	0.51 ± 0.03	0.85 ± 0.02	0.54 ± 0.03	0.68 ± 0.03	4.6 ± 0.1	0.89 ± 0.02	0.52 ± 0.02	5.2 ± 0.11

Note. The binding was evaluated as the BSA-coupled peptides to directly retain core particles. Results were given as O.D. sample/cut-off value being twofold the mean O.D. value obtained with BSA alone. Values reported here represent the mean O.D. sample/cut-off and standard deviations of 3 independent experiments.

or to the different origins of the HBV core particles: recombinant *Escherichia coli*-derived HBV core particles were used in the previous study, whereas we used human liver-derived HBV core particles. Our results are consistent with the data reported by Bruss and Thomssen (*7*), who tested deletion mutants for their ability to support virion maturation. They showed that the 17 C-terminal amino acids of pre-S1 play a key role in virion morphogenesis. Our study suggests that the 13 C-terminal amino acids of pre-S1 (PLSPPLRNTHPQA) are a target for HBV core particles during virion maturation. Interestingly, 8 (62%) of these 13 amino acids are identical (PPTPPLRDTHPHL) in the woodchuck hepatitis virus (WHV) L protein (*21*), which is able to replace the HBV L protein in HBV maturation (*22*). The pre-S2 domain, as represented by the L5 peptide, did not show any binding affinity to HBV core particles.

S domains: Peptide S1 (amino acids 31 to 55 of the cytosolic loop of S) did not bind to HBV core particles. Peptide S2 (amino acids 56 to 80) bound to HBV cores in both inhibition immunoassay and direct binding assay (Tables 1 and 2). Thus a S domain binds to HBV core particles and may contribute to the virion morphogenesis. This may explain why HBV virions lacking C-terminal pre-S1 sequences have been observed in sera of some patients (*23*). Possibly S domain interaction with the HBV core is sufficient for some virions to be enveloped and secreted. This additional interaction with the HBV core may be provided either by the cytolosic loop of S or by the i-pre-S form of the L protein which exposes the N terminal sequence of S up to signal II on the cytosolic side of ER. However, a specific interaction of S with the HBV core may explain why HBV mutants lacking the i-pre-S form of L are able to envelop and secrete low levels of virions (*24*). Furthermore, it has been reported that transfected murine fibroblastic LTK cells produce and secrete HBV-like particles although they barely express the L protein (*25*). A comparison of the amino acid sequence of S between positions 56 to 80 with the corresponding region of the WHV S protein (*21*) shows a 72% identity: QTCKHLPTSCPPTCNGFRWMYLRRF. This may be of importance, since WHV S protein is able to substitute for its HBV conterpart in cooperation with HBV L for virion maturation (*22*).

Deletions in the cytosolic loop of S hamper particle assembly and secretion (*26*). *In vitro* peptide/core binding assays are thus very convenient alternatives to cell culture for exploring the function of this HBV envelope protein using deletion mutants. We could not, however, investigate the potential core binding affinity of the type I signal, also exposed at the cytosolic side of ER in the i-pre-S form of L, because the peptide covering this domain was too hydrophobic to be solubilized in the conditions used in our assays.

In conclusion, our data suggest that both pre-S1 and S domains of HBV envelope proteins have binding affinities to HBV core particles *in vitro.* This dual interaction of the HBV core with HBV envelope protein domains may be required for the virion morphogenesis.

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